280967

STIC-ILL

From: Wessendorf, Theresa
Sent: Thursday, January 27, 2000 4:28 PM
To: STIC-ILL
Subject: 09/229,751

Please forward:
1. Rapid Comm. Mass Spectrom. 10, 29,-35 (1996)Wunschel.
2. J. clin. Micro. 27, 1257-1261 (1989) Starnach. Issue 6
3. Molec. Microbiol. 3, 843-49 (1989).
thanksWessendorf, Theresa

t. Wessendorf
Art Unit 1627
Rm. 2B17, mailroom 2C01
Phone 308-3967

MIC
QR46.J87

JOURNAL OF CLINICAL MICROBIOLOGY, June 1989, p. 1257–1261
0095-1137/89/061257-05$02.00/0

Vol. 27, N . 6

Species-Specific Detection of *Legionella pneumophila* in Water by DNA Amplification and Hybridization

MICHAEL N. STARNBACH,* STANLEY FALKOW, AND LUCY S. TOMPKINS

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305

Received 16 December 1988/Accepted 28 February 1989

A sensitive detection system specific for *Legionella pneumophila* in water was developed. This system is based n amplification of a chromosomal DNA sequence from *L. pneumophila* by the polymerase chain reaction, followed by detection of the amplified product by hybridization of a radiolabeled oligodeoxynucleotide. After 35 cycles of amplification, a water sample which had been seeded with 35 CFU of *L. pneumophila* contained sufficient amplified DNA to be detected on dot blots. Bacteria of other genera tested did not generate positive signals under these conditions. Application of this technique to environmental water samples may help identify the natural reservoirs of nosocomial and community-acquired *L. pneumophila* infections.

Legionella pneumophila is recognized as an important cause of atypical pneumonia. Although *L. pneumophila* usually causes a small number of community-acquired respiratory infections, the number of hospital-acquired cases in the United States is rising. Presumably, this increasing rate of Legionnaires disease in hospitalized patients is primarily related to the growing number of susceptible hosts. Among the known risk factors for infection are renal dialysis, organ transplantation, alcohol consumption, corticosteroid or other immunosuppressive therapy, smoking and chronic lung disease, cancer, and other chronic underlying illnesses. Although many clinical aspects of the disease are well understood, the reservoirs for *L. pneumophila* and the modes of transmission that occur in the hospital setting are poorly understood.

Outbreaks of nosocomial legionellosis have been attributed to exposure of susceptible individuals to aerosols of contaminated water. Building air-exchange equipment and whirlpool baths have been epidemiologically linked to community and hospital epidemics (1). Potable water has also been linked to outbreaks (14). Transmission from contaminated potable water has been ascribed to showers (15), contaminated respiratory therapy equipment, and possibly ingestion of tap water (1). While cultivation of *L. pneumophila* is the standard method of detection, it has not always been possible to isolate viable bacteria from suspected water sources. Several groups have reported detection of legionellae by using DNA probes. Grimont et al. (7) selected and pooled endonuclease restriction fragments of chromosomal DNA from an isolate of *L. pneumophila*, radiolabeled the fragments, and used the pooled fragments to detect legionella which had been seeded onto nitrocellulose membranes and lysed in situ. This method could detect no fewer than 10^5 CFU. Engleberg et al. (6) reported a sensitivity of 5×10^4 organisms using another DNA probe to detect legionellae in murine lung tissue homogenates. A commercially available kit (4) also employs a DNA probe to detect legionellae.

All the existing probes require relatively large quantities of *Legionella* DNA f r detection by hybridizati n. This precludes their use f r directly detecting legionellae in environmental or potable water sources suspected f contamination. The development of the polymerase chain reaction (13) and the incorporation of the thermostable *taq* DNA polymerase into this process (12) permit the automated amplification of target DNA sequences. It is estimated that the process is capable of selectively amplifying a particular sequence of DNA by a factor of 10^6 (12). Thus, the polymerase chain reaction can amplify a single copy of the target such that it is well within the range of traditional DNA probe detection levels. In this study, we investigated the feasibility of using the polymerase chain reaction to amplify a specific *L. pneumophila* chromosomal DNA fragment in water samples. Following amplification, a DNA probe was used to detect the amplified product.

* Corresponding author.

MATERIALS AND METHODS

Growth of *L. pneumophila* and cloning of chromosomal DNA fragment. *L. pneumophila* strains were grown in a liquid medium consisting of 15 g of yeast extract (Difco Laboratories, Detroit, Mich.) and 2 g of ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid] per liter, adjusted to pH 6.9 with KOH, and supplemented with 0.4 g of L-cysteine after autoclaving. The number of CFU per milliliter was determined by serial dilution and plating on buffered charcoal-yeast extract agar (BBL Microbiology Systems, Cockeysville, Md.).

Chromosomal DNA from *L. pneumophila* serogroup 1 (Stanford L6/s) (16) was isolated by the method of Roussel and Chabbert (11), digested with both *Eco*RI and *Hin*dIII, and applied to a 1% low-melting-point agarose gel (SeaPlaque; FMC Corp., Marine Colloids Div., Rockland, Maine) containing 0.5 μg of ethidium bromide per ml. After electrophoresis, a segment of the lane containing DNA fragments between 800 and 1,200 base pairs (bp) in size was cut from the gel. These fragments were extracted from the gel (10) and ligated into plasmid pBS− (Stratagene, La Jolla, Calif.) which had been digested with both *Eco*RI and *Hin*dIII. The ligation mix was transformed into *Escherichia coli* XL-1 Blue (Stratagene). Six of the resulting recombinant clones were screened for an insert which hybridized to *L. pneumophila* chromosomal DNA and did not hybridize to DNA f ther bacterial genera (by Southern analysis, see method below). One cl ne, pLEG101, which met these criteria contained an 800-bp *Eco*RI-*Hin*dIII insert. The same insert was als cl ned into plasmid pBS+ (Stratagene) t allow for eventual sequencing of both complementary strands of the *L. pneumophila* DNA fragment. The resultant plasmid, pLEG102, was transf rmed int strain XL-1 Blue.

Southern analysis of bacterial DNA. Bacterial chromosomal DNA was cut with both *Eco*RI and *Hin*dIII, and equivalent amounts were applied to a 1% agarose gel. After electrophoresis, the DNA was transferred to a nitrocellulose membrane and baked under vacuum for 2 h at 80°C (9). The membrane was prehybridized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–0.001 M EDTA–1× Denhardt solution–50% formamide for 18 h at 37°C (9). The 800-bp DNA fragment from pLEG102 was purified from a low-melting-point agarose gel as described above, labeled with [α-^{32}P]dCTP by nick translation (9), and boiled for 5 min. The probe was added to the prehybridization buffer along with 100 μg of sheared, denatured calf thymus DNA per ml. The probe was hybridized for 24 h at 37°C. After hybridization, the blot was washed in 5× SSC–0.1% SDS at 65°C for 1 h, rinsed twice in 2× SSC at room temperature, allowed to dry, and exposed to Kodak XAR-5 film.

DNA sequence analysis. Single-stranded DNA was recovered from pLEG101 and pLEG102 by use of the M13 helper bacteriophage VCS-M13 (Stratagene). Using the appropriate primers (T7 and T3; Stratagene) and the Sequenase sequencing reagents (U.S. Biochemical Corp., Cleveland, Ohio), 150 bp of nucleotide sequence at each end of the 800-bp insert was determined.

Amplification of samples. Bacterial suspensions (72.5 μl) in sterile distilled water were placed in 0.6-ml microcentrifuge tubes. To lyse the bacteria and release the chromosomal DNA, the tubes were frozen in dry ice for 5 min, boiled for 5 min, frozen again, then boiled again. Each tube was placed on wet ice, and 27 μl of concentrated amplification mixture was added such that each tube contained the following (12): 50 mM KCl; 10 mM Tris-chloride (pH 8.3); 1.5 mM $MgCl_2$; 0.01% gelatin; 200 μM each dATP, dCTP, dGTP, and TTP; 1.0 μM oligodeoxynucleotide LEG-1; and 1.0 μM oligodeoxynucleotide LEG-2. A 2.5-U portion (0.5 μl) of *taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was then added to each tube so that the final sample volume was 100 μl. Samples to be amplified were placed in a programmable thermal cycler and subjected to 35 cycles of amplification. One cycle consisted of 1 min at 93°C, 1 min at 55°C, and 1.5 min at 74°C. After the final cycle, the tubes were incubated for an additional 7 min at 74°C.

Dot blots of amplified product. Samples for blotting were removed from the reaction tubes and boiled for 10 min, and 30 μl was spotted onto a nitrocellulose membrane. The nitrocellulose was baked under vacuum at 80°C for 2 h and incubated in a prehybridization buffer of 6× SSC, 5× Denhardt solution, and 0.1% SDS (9) for 2 h at 42°C. The probe for the dot blots was a 25-bp oligodeoxynucleotide, LEG-3 (5'-GTCCGTTATGGGGTATTGATCACCA-3'), end labeled with [γ-^{32}P]dATP by using polynucleotide kinase (3). This probe was added to a hybridization buffer containing 6× SSC, 1× Denhardt, 0.1% SDS, and 100 μg of sheared and denatured calf thymus DNA per ml (9). The blot was incubated in this hybridization mixture for 6 h at 42°C. After hybridization, the blot was washed twice with 6× SSC–0.1% SDS at 55°C for 30 min each time, rinsed briefly with 6× SSC at room temperature, allowed to dry, and exposed to Kodak XAR-5 film.

Materials. Chemical reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.). Restriction endonucleases and other DNA-modifying enzymes were supplied by Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Oligodeoxynucleotides were synthesized by Operon Technologies (San Pablo, Calif.).

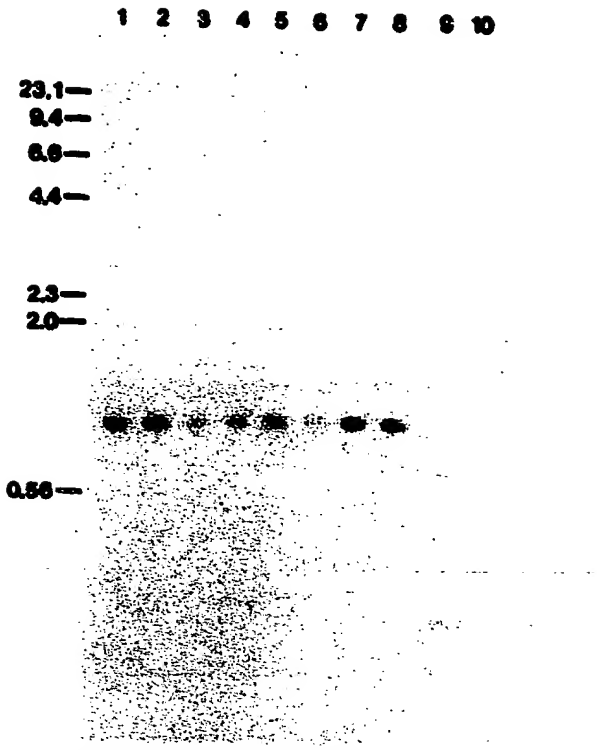

FIG. 1. Southern blot of chromosomal DNA from various bacteria probed with the 800-bp *Eco*RI-*Hin*dIII insert. All lanes contain chromosomal DNA digested with *Eco*RI and *Hin*dIII. DNA was from *L. pneumophila* (Stanford Lu/s) (16) (lane 1); *L. pneumophila* (Philadelphia 1) (Centers for Disease Control) (lane 2); *L. pneumophila* (Philadelphia 1s) (Centers for Disease Control) (lane 3); *L. pneumophila* (serogroup 2, SG1-C2) (16) (lane 4); *L. pneumophila* (serogroup 6, Stanford University Hospital (lane 5); *L. pneumophila* (serogroup 8, SG8) (16) (lane 6); *L. pneumophila* (Stanford L11, Stanford University Hospital (lane 7); *L. pneumophila* (Naples) (16) (lane 8); *E. coli* (HB101) (11) (lane 9); and *P. aeruginosa* (Stanford University Hospital) (lane 10). Positions and sizes (in kilobases) of λ DNA digested with *Hin*dIII are shown on the left.

RESULTS

Identification of *L. pneumophila*-specific DNA probe. A chromosomal DNA fragment from *L. pneumophila* was identified for use in the development of a species-specific DNA amplification and hybridization detection system. The selected fragment was cloned into pBS− and pBS+ and named pLEG101 and pLEG102, respectively. To determine the specificity of the cloned 800-bp *L. pneumophila* insert, it was used as a probe in the Southern analysis of chromosomal DNA from a variety of bacteria (Fig. 1). All *L. pneumophila* serogroups tested contained an 800-bp *Eco*RI-*Hin*dIII fragment which hybridized to the 800-bp probe. No hybridization was apparent in lanes containing chromosomal DNA from *E. coli* or *Pseudomonas aeruginosa*.

Amplification of *L. pneumophila* samples. The polymerase chain reaction requires the use of two oligonucleotides which flank the region of DNA to the amplified. Sequence analysis of the 800-bp fragment was done so that appropriate oligodeoxynucleotides could be chosen. Two 19-mer oligodeoxynucleotides were synthesized which were complementary to 19 bases near the termini of the fragment and which bound to opposite strands of the DNA. These two oligodeoxynucleotides were LEG-1 (5'-GTCATGAGGAATCTCGCTG-3') and LEG-2 (5'-CTGGCTTCTTCCAGCTTCA-3').

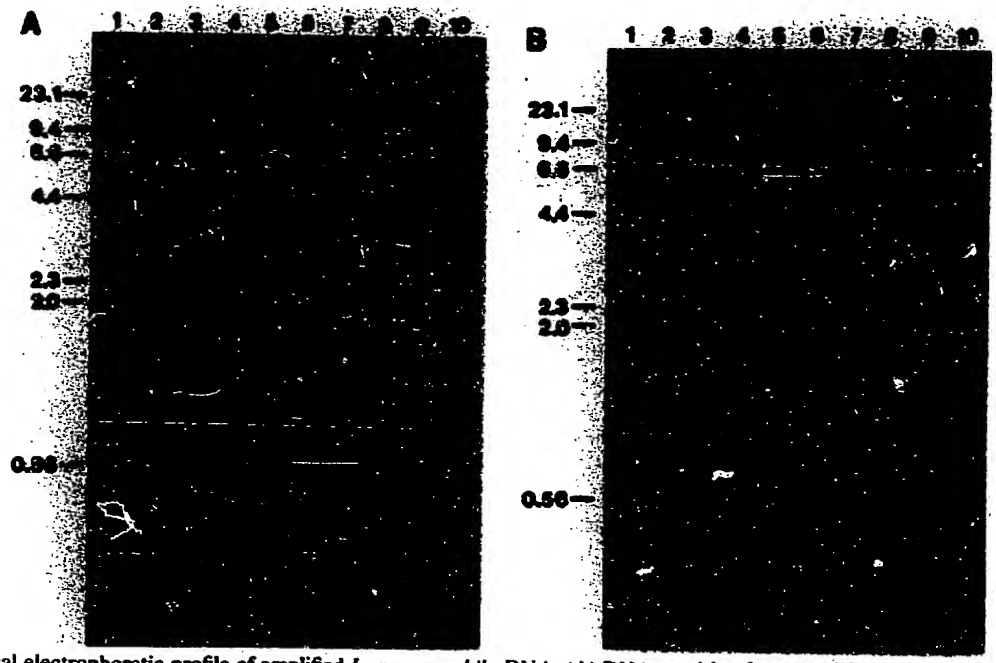

FIG. 2. Typical electrophoretic profile of amplified *L. pneumophila* DNA. (A) DNA resulting from amplification of samples seeded with the following (CFU): 3.5×10^7 (lane 1), 3.5×10^6 (lane 2), 3.5×10^5 (lane 3), 3.5×10^4 (lane 4), 3.5×10^3, (lane 5), 350 (lane 6), 35 (lane 7), 3.5 (lane 8), 0.35 (lane 9), and 0 (lane 10). Numbers represent the average number of *L. pneumophila* CFU which would be present in multiple samples as determined by dilution of a bacterial concentrate. (B) Replicate samples which did not undergo amplification by thermal cycling. Positions and sizes (in kilobases) of λ DNA digested with *Hin*dIII are shown to the left of each panel.

To determine the sensitivity of the detection system, we amplified samples of *L. pneumophila* containing from 10^7 CFU to fewer than 1 CFU. Initially, *L. pneumophila* (Stanford L6/s) (16) was grown to a concentration to 10^8 to 10^9 CFU/ml. Bacteria from 1.0 ml of this culture were pelleted in an Eppendorf microcentrifuge for 2.5 min, suspended in 1.0 ml of sterile distilled water, and serially diluted. In addition, a tube containing no cells was included as control. Each tube was prepared in duplicate. One sample was amplified, and the other was treated in exactly the same manner except it did not undergo the thermal cycling which permits amplification.

Analysis of amplified product. To characterize the product of the amplification reaction, we removed 2 μl of the 100-μl reaction mixture from each tube after amplification. These samples were applied to 1% agarose gels containing 0.5 μg of ethidium bromide per ml and subjected to electrophoresis. Figure 2A shows a typical electrophoretic profile of the DNA after 35 cycles of the polymerase chain reaction. Samples containing 350 CFU or more displayed a band corresponding to 700 bp. This was the size of the expected amplification product, slightly smaller than the 800-bp cloned *L. pneumophila* chromosomal fragment. When amplification was carried out for 35 cycles, samples containing fewer than 350 CFU did not show a band on ethidium bromide-stained gels. As expected, no amplified product was found in samples which did not undergo thermal cycling (Fig. 2B).

Hybridization of a radiolabeled probe is the most sensitive method to detect specific DNA sequences. Thus, dot blots of the amplified product were prepared and probed with a radiolabeled oligodeoxynucleotide, LEG-3. LEG-3 was complementary to sequences between the two amplification primers, near, and complementary to, the same strand as LEG-2. In the dot blot shown in Fig. 3, the LEG-3 probe hybridized with amplified samples which contained 35 CFU or more of *L. pneumophila*. Samples containing fewer than 35 bacteria or those which were not amplified did not hybridize with the probe. Dot blots reproducibly detected samples containing more than 35 CFU.

Specificity of detection system. Water samples seeded with between 10^7 and 10^9 CFU of bacteria of other genera were prepared, amplified, and blotted in the same manner as the *L. pneumophila* samples. Species tested were *Aeromonas hydrophila*, *Citrobacter diversus*, *Enterobacter agglomerans*, *Escherichia coli*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Providencia stuartii*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens*, *Shigella flexneri*, *Vibrio parahaemolyti-*

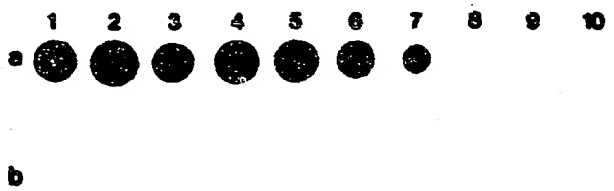

FIG. 3. Typical dot blot of amplified samples probed with LEG-3 oligodeoxynucleotide. Row a shows DNA resulting from amplification of samples seeded with the following (CFU): 3.5×10^7 (lane 1), 3.5×10^6 (lane 2), 3.5×10^5 (lane 3), 3.5×10^4 (lane 4), 3.5×10^3 (lane 5), 350 (lane 6), 35 (lane 7), 3.5 (lane 8), 0.35 (lane 9), and 0 (lane 10). Numbers represent the average number of *L. pneumophila* CFU which would be present in multiple samples as determined by dilution of a bacterial concentrate. Row b shows replicate samples which did not undergo amplification by thermal cycling.

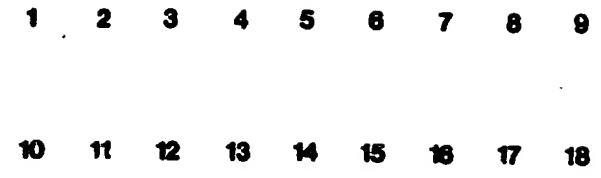

FIG. 4. Dot blot showing hybridization of LEG3 to other genera of bacteria after amplification. Samples: 1, *L. pneumophila*; 2, *A. hydrophila*; 3, *C. diversus*; 4, *Enterobacter agglomerans*; 5, *Escherichia coli*; 6, *H. alvei*; 7, *K. pneumoniae*; 8, *M. morganii*; 9, *Proteus mirabilis*; 10, *Providencia stuartii*; 11, *Pseudomonas aeruginosa*; 12, *Salmonella typhimurium*; 13, *Serratia marcescens*; 14, *Shigella flexneri*; 15, *V. parahaemolyticus*; 16, *Y. enterocolitica*; 17, human feces; and 18, sterile distilled water.

cus, and *Yersinia enterocolitica*. Tubes containing 4×10^{-4} g of human feces were also subjected to amplification. Such a fecal sample has been estimated to contain approximately 4×10^7 bacteria and represents a diverse array of microorganisms (2). Amplification of water samples seeded with bacteria of other genera, including the human fecal sample, did not yield a product which hybridized with the LEG-3 probe (Fig. 4).

To test the reactivity of the system with other members of the *Legionella* genus, samples containing purified chromosomal DNA corresponding to 10^7 and 10^8 CFU of *L. pneumophila*, *L. dumoffii*, *L. feelei*, *L. jordanis*, and *L. micdadei* were amplified and probed in the same manner as the bacterial suspensions. Only the *L. pneumophila* sample produced a positive signal (data not shown).

DISCUSSION

Cultivation of *L. pneumophila* from clinical materials has been considered the standard against which other detection methods are compared. However, many investigators have been unable to confirm *Legionella* contamination of environmental and potable water samples by cultivation and have failed to pinpoint the reservoirs and mode of transmission of several nosocomial *Legionella* strains (16). Attempts to cultivate these strains on a variety of media and to isolate them by inoculation of guinea pigs have failed despite the ease with which the strains can be grown from clinical specimens on routine *Legionella* media (16). Although the commercially available *Legionella* DNA probe kit has reasonable sensitivity when applied to respiratory tract specimens (5), we have found it to be quite insensitive for detecting *Legionella* species in potable water samples, even some that were culture positive (unpublished observations). Amplification of target DNA sequences before probing offers a more sensitive alternative to detect a small number of viable, cultivatable bacteria and even so-called "noncultivatable" or "nonviable" forms (8). We therefore developed a species-specific *L. pneumophila* detection system based on enzymatic amplification of target sequences followed by hybridization of a radiolabeled DNA probe.

The DNA amplification and probe system described in this study is specific for *L. pneumophila*. No significant cross-reactivity with other *Legionella* species or other bacterial genera has yet been observed. Furthermore, we could detect as few as 35 CFU of *L. pneumophila*, a far greater sensitivity than observed with any previously described technique. Theoretically, a single bacterium could be detected with 60 cycles of amplification; however, under these conditions, we often obtained positive signals on dot blots from samples which were not known to contain *L. pneumophila* bacteria or DNA. These signals likely resulted from laboratory or environmental contamination of the samples with *L. pneumophila* bacteria or DNA. Therefore, we reduced the number of amplification cycles to produce a highly specific but slightly less sensitive test.

Although the method is specific only for *L. pneumophila*, probes specific for other pathogenic legionellae could similarly be constructed. In instances in which detection of multiple species is desirable, the oligodeoxynucleotides could be mixed and amplification carried out simultaneously in the same tube. Mixed radiolabeled probes would be used for detection.

We expect that the application of DNA probes to environmental and potable water samples, coupled with specific amplification of target sequences, will contribute to the advancement of *Legionella* epidemiology. Before this technology can be applied to water samples, however, there are many factors that must be controlled. First, owing to the sensitivity of the polymerase chain reaction, precautions must be taken to prevent contamination of glassware, buffers, and other solutions with viable *Legionella* species that might be found in water baths, distilled water systems, etc., or with *Legionella* DNA. Second, the results of probing culture-negative water samples must be coupled with epidemiological or clinical information whenever possible.

In summary, application of a DNA amplification and hybridization detection system to environmental water samples was found to be a sensitive method that theoretically could detect legionellae undetectable by other means. Use of such systems may ultimately lead to the identification and decontamination of relevant sites in the hospital and thus may promote a decline in nosocomial infection rate.

ACKNOWLEDGMENTS

We thank Nancy Troup and Fred Quinn for providing *Legionella* strains and chromosomal DNA from those strains. Denise Monack generously provided biological material. We appreciate critical reading of the manuscript by Catherine Lee and David Relman.

This work was supported by Public Health Service grant AI23796 from the National Institutes of Health. M.N.S. was supported by Public Health Service training grant AI07328 from the National Institutes of Health.

LITERATURE CITED

1. **Broome, C. V.** 1983. Epidemiologic assessment of methods of transmission of legionellosis. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A 255:52–57.
2. **Davis, B. D., R. Dulbecco, H. N. Eisen, and H. S. Ginsberg.** 1980. Microbiology, including immunology and molecular genetics. 3rd ed. Harper and Row, Publishers, Inc., Hagerstown, Md.
3. **Davis, L. G., M. D. Dibner, and J. F. Battery.** 1986. Basic methods in molecular biology. Elsevier Science Publishing, Inc., New York.
4. **Edelstein, P. H.** 1986. Evaluation of the Gen-Probe DNA probe for the detection of legionellae in culture. J. Clin. Microbiol. 23:481–484.
5. **Edelstein, P. H., R. N. Bryan, R. K. Enns, D. E. Kohne, and D. L. Kacian.** 1987. Retrospective study of Gen-Probe rapid diagnostic system for detection of legionellae in frozen clinical respiratory tract samples. J. Clin. Microbiol. 25:1022–1026.
6. **Engleberg, N.C., C. Carter, P. Demarsh, D. J. Drutz, and B. I. Eisenstein.** 1986. A *Legionella*-specific DNA probe detects organisms in lung tissue homogenates from intranasally inoculated mice. Isr. J. Med. Sci. 22:703–705.
7. **Grimont, P. A. D., F. Grimont, N. Desplaces, and P. Tchen.**

1985. DNA probe specific for *Legionella pneumophila*. J. Clin. Microbiol. 21:431–437.
8. **Hussong, D., R. R. Colwell, M. O'Brien, E. Weiss, A. D. Pearson, R. M. Weiner, and W. D. Burge.** 1987. Viable *Legionella pneumophila* not detectable by culture on agar media. Bio/Technology 5:947–950.
9. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Ogden, R. C., and D. A. Adams.** 1987. Electrophoresis in agarose and acrylamide gels. Methods Enzymol. 152:61–87.
11. **Roussel, A. L., and Y. A. Chabbert.** 1978. Taxonomy and epidemiology of gram-negative bacterial plasmids studied by DNA-DNA hybridization in formamide. J. Gen. Microbiol. 104:269–276.
12. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491.
13. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification f β-globin genomic sequences and restricti n site analysis f r diagn sis of sickle cell anemia. Science 230:1350–1354.
14. **Stout, J., V. L. Yu, R. M. Vickers, J. Zuravieff, M. Best, A. Brown, R. B. Yee, and R. Wadowsky.** 1982. Ubiquitousness f *Legionella pneumophila* in the water supply of a hospital with endemic Legionnaires' disease. N. Engl. J. Med. 306:466–468.
15. **Tobin, J. O., J. Beare, M. S. Dunnill, S. Fisher-Hoch, M. French, R. G. Mitchell, P. J. Morris, and M. F. Muers.** 1980. Legionnaires' disease in a transplant unit: isolation of the causative agent from shower baths. Lancet ii:118–121.
16. **Tompkins, L. S., N. J. Troup, T. Woods, W. Bibb, and R. M. McKinney.** 1987. Molecular epidemiology of *Legionella* species by restriction endonuclease and alloenzyme analysis. J. Clin. Microbiol. 25:1875–1880.